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Removal of glycosaminoglycans from cultures of human skin fibroblasts

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Early-passage human skin fibroblasts were grown as monolayers for 2–3 days in minimum essential medium containing [^{35}S]sulphate, [^{3}H]glucosamine, [^{3}H]fucose, [^{3}H]proline or [^{3}H]leucine to label proteoglycans, glycoproteins or collagen and other proteins. A crude enzyme preparation obtained from a supernatant from sonicated freeze-dried *Flavobacter heparinum* was added to the cell monolayers. This treatment removed most of the ^{35}S-labelled glycosaminoglycans, with no appreciable removal of the ^{3}H-labelled proteins or ^{3}H-labelled glycoproteins. The cells remained attached and viable as a monolayer. The formation of ^{35}S-labelled glycosaminoglycans was examined after pretreating cultures with crude *F. heparinum* enzyme, followed by addition of fresh growth medium containing [^{35}S]sulphate. The *F. heparinum* enzyme did not significantly alter the amount or type of ^{35}S-labelled glycosaminoglycans produced. Thus *F. heparinum* enzyme can be used to provide cultured-cell monolayers depleted of surface glycosaminoglycans. These cells remain attached, viable and subsequently synthesize normal amounts and type of glycosaminoglycans.

Proteoglycans are produced by most and perhaps all mammalian cells grown in culture. These compounds have been located on the cell surface, in extracellular matrix, in growth media and intracellularly. Their physiological function is not known, but might relate to cell proliferation, adhesion, aggregation, interaction with matrix substances etc. It has been suggested that the presence of glycosaminoglycans or proteoglycans in cell matrix helps to control cell growth and differentiation (Toole & Gross, 1971) and may have an effect on further production of proteoglycans (Nevo & Dorfman, 1972; Wiebkin & Muir, 1977).

Direct information regarding function of cellular and cell-layer proteoglycans might be obtained if a simple method for selective removal of proteoglycans or glycosaminoglycans were available. The effect on cell behaviour of specific proteoglycan or glycosaminoglycan loss from the cell surface or cell matrix could then be evaluated. Matrix and cell-surface proteoglycans can be effectively removed by trypsin (Kraemer, 1971*a*,*b*; Kleinman *et al.*, 1975). However, trypsin also removes other cell components such as proteins and glycoproteins, and lifts the cells from a monolayer. Chondroitin ABC lyase has been used to remove chondroitin sulphate from cells in culture (Saito & Uzman, 1971; Perkins *et al.*, 1979), but this enzyme does not remove heparan sulphate, which is a significant component of the cell-layer matrix and is the major glycosaminoglycan component of the cell surface (Kraemer, 1971*a*,*b*; Kraemer & Smith, 1974; Kleinman *et al.*, 1975). A partially purified heparinase (containing heparanase activity) from *Flavobacter heparinum* (Buonassisi & Root, 1975) and a platelet heparanase (Wasteson *et al.*, 1977) have been used on endothelial-cell cultures. These enzymes effectively removed heparan sulphate, while leaving chondroitin sulphate. The possible removal of other cell-layer materials and the viability of the cells after these treatments have not been described. No information has been provided regarding the effects of selective proteoglycan or glycosaminoglycan removal from cell cultures on subsequent synthesis of proteoglycans or glycosaminoglycans.

The purified heparinase and the platelet enzyme are difficult to obtain in quantity. Neither enzyme removes chondroitin sulphate. For these reasons we have utilized a crude easily prepared enzyme mixture from *F. heparinum* for removal of all types of glycosaminoglycans from cell-surface and cell-layer

Abbreviations used: ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluc-4-enepyranosyluronic acid)-4-*O*-sulpho-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluc-4-enepyranosyluronic acid)-6-*O*-sulpho-D-galactose.

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matrix. We have determined the effect of this preparation on cells, its specificity for glycosaminoglycan removal and the effect of this removal on subsequent synthesis of glycosaminoglycans.

Materials

Sodium [^{35}S]sulphate (sp. radioactivity 0.8 Ci/mmol), [^{3}H]proline (sp. radioactivity 5 Ci/mmol), [^{3}H]leucine (sp. radioactivity 118 Ci/mmol), [^{3}H]-fucose (sp. radioactivity 3 Ci/mmol) and [^{3}H]glucosamine (sp. radioactivity 18.8 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). Eagle's minimum essential medium supplemented with 10% foetal calf serum (Gibco, Grand Island, NY, U.S.A.) was utilized as the routine growth medium. Sulphate-poor minimum essential medium, in which $MgCl_2$ was substituted for $MgSO_4$, was used in experiments utilizing [^{35}S]sulphate. Phosphate-buffered saline (138 mM-NaCl, 2.7 mM-KCl, 8.1 mM-Na_2HPO_4 and 1.47 mM-KH_2PO_4) with 0.4 mM-$MgSO_4$ and 0.68 mM-$CaCl_2$ was used as the major stock buffer unless otherwise specified. Bacterial chondroitin AC lyase and chondroitin ABC lyase were purchased from Miles Laboratories (Elkhart, IN, U.S.A.). Collagenase was purchased from Advanced Biofactors Corp. (Lynnbrook, NY, U.S.A.). Trypsin, neuraminidase and bovine submaxillary mucin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *F. heparinum* was grown in large quantities by the New England Enzyme Center (Boston, MA, U.S.A.) by the methods of Linker & Hovingh (1972). Purified heparinase was supplied by Dr. A. Linker.

Skin-punch biopsies (3–4 mm) from normal 20–35-year-old male subjects were cut into 0.5–1.0 mm explants, placed in 25 cm^2 Corning flasks and allowed to grow in minimum essential medium containing 10% foetal calf serum and 50 mg of gentamycin/ml, at 37°C in a humidified CO_2/air (1:19) environment. Fibroblast outgrowths from these explants were harvested with 0.25% trypsin in phosphate-buffered saline and passed to new flasks. Once the lines were established, cell suspensions of early passages in complete medium containing 10% dimethyl sulphoxide were banked in liquid N_2. Ampoules of the frozen cell suspension could then be thawed and plated as needed.

Methods

Enzyme activity

For the preparation of crude *F. heparinum* enzyme, 100 mg of freeze-dried *F. heparinum* in 4 ml of 0.5 M-sodium acetate at pH 7.0 was sonicated for 60 s, centrifuged at 30000 *g* for 20 min and the active supernatant was filtered through a 0.22 nm Millipore filter. The supernatant stored at −20°C retained activity for at least 6 months. This crude preparation contains chondroitin lyase, dermatanase, heparinase and heparanase (heparitinase) activities (Korn, 1957) and can thus degrade all the sulphated glycosaminoglycans produced by skin fibroblasts. The initial products of degradation by these enzymes are oligosaccharides. Activity can be measured by an increase in A_{232}, owing to an unsaturated double bond introduced during the cleavage between the hexosamines and uronic acids (Linker & Hovingh, 1972; Suzuki, 1972). Complete exclusive polysaccharidase action would result in disaccharides as products. However, the crude *F. heparinum* preparation also contains sulphatases and glycuronidases, which will degrade some oligosaccharides further (Linker & Hovingh, 1965, 1972). Activity of the crude *F. heparinum* enzyme was expressed as μmol of unsaturated double bond appearing from a heparin substrate/h. Chondroitin AC lyase and chondroitin ABC lyase were also monitored at 232 nm in the same fashion with a mixture of chondroitin 4-sulphate and chondroitin 6-sulphate as substrates. Units were expressed similarly as μmol/h and not as μmol/min as described by Suzuki (1972). Thus 60 units equal 1 'Suzuki' unit.

The possible presence of collagenase activity was assayed by examining for release of ^{14}C-labelled products from [^{14}C]collagen (a gift from Dr. Barbara D. Smith) that had been labelled *in vivo* and isolated from guinea-pig skin (Nagai *et al.*, 1966). The possible presence of neuraminidase activity was estimated by incubating 1.5 units of crude *F. heparinum* enzyme with 0.2% bovine submaxillary-gland mucin (Sigma), in either 0.5 M-sodium acetate buffer at pH 5.1 or in phosphate-buffered saline in a total volume of 1.0 ml. Release of sialic acid was measured by the thiobarbituric acid method (Warren, 1959). Release of sialic acid from mucin with neuraminidase (*Clostridium perfringens*; type VI, Sigma) added to the same preparations was used as a control. Non-specific proteinase activity of the crude *F. heparinum* preparation was measured by incubating several concentrations of the preparation with 1% casein (Fisher Scientific, Pittsburgh, PA, U.S.A.) at 37°C for 0.5 h in 0.1 M-potassium phosphate buffer, pH 7.6. Undegraded protein was precipitated with 5% trichloroacetic acid and degraded unprecipitated material was assayed by determination of A_{280} (Laskowski, 1955). *F. heparinum* preparations were also assayed for proteinase activity similarly with heat-denatured (50°C for 10 min) ^{14}C-labelled collagen in 0.05 M-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.9, after incubation at 37°C for 40 min (Nagai *et al.*, 1966).

Cell cultures and fractionation procedures

Early passages of fibroblasts were seeded at a density of 3×10^5–5×10^5 cells/25 cm^2 flask in 5 ml of standard growth medium. After incubation for 2–3 days at 37°C, fresh medium with 10% foetal calf serum [antibiotic-free and sulphate-poor (0.08 mM-SO_4^{2-})] was added with the appropriate labelled precursors and incubated for 48 h. After removal of the medium, the cell monolayer was washed five or six times with 5 ml of iced phosphate-buffered saline containing 100 mg of $CaCl_2$ and 100 mg of $MgSO_4$ per litre until [^{35}S]sulphate and/or ^{3}H-labelled precursor was no longer washed off the cells. Plated cultures were then incubated for various periods with either (*a*) 0.5 ml of phosphate-buffered saline or phosphate-buffered saline plus boiled enzyme as a control, (*b*) chondroitin AC lyase in 0.5 ml of phosphate-buffered saline, (*c*) chondroitin ABC lyase in 0.5 ml of phosphate-buffered saline, (*d*) *F. heparinum* enzyme in 0.5 ml of phosphate-buffered saline or (*e*) 0.25% trypsin in 0.5 ml of phosphate-buffered saline (Ca^{2+}- and Mg^{2+}-free). Each of these solutions was removed and kept for analysis and identification. When the above treatments were performed sequentially, cells were washed once with phosphate-buffered saline between treatments and the washings pooled with the prior incubation solution. After enzyme treatment or phosphate-buffered saline control, the final treatment was always 0.5 ml of 0.25% trypsin in phosphate-buffered saline (Ca^{2+}- and Mg^{2+}-free), which quantitatively lifted the fibroblasts from the plates. The cell suspensions were then centrifuged, which resulted in a supernatant (trypsinate fraction) and a cell pellet. The trypsinate fraction includes glycosaminoglycans derived from the solubilized matrix and from the cell surface. The flasks were then treated with 0.5 ml of 0.5 M-NaOH for removal of radioactivity remaining on the plates. Cell populations were determined from portions of resuspended cell pellets, and intracellular glycosaminoglycans were analysed after three cycles of freezing and thawing of the pellet.

Viability studies were performed by lifting cells with 0.25% trypsin, counting and replating. The plated cells were lifted again with trypsin before cell division and counted. The medium was examined for unattached cells. Plating efficiency was measured by the expression:

$$\frac{\text{no. of cells lifted by trypsin} \times 100}{\text{no. of cells plated}}$$

In addition, viability was measured by the ability of cells to exclude Trypan Blue (Patterson, 1979).

Analysis of products

The difference in radioactivity released by an enzyme treatment from that of the phosphate-buffered saline control was used to represent the amount of ^{35}S-labelled glycosaminoglycan or ^{3}H-labelled material released at any specific step. For identification, ^{35}S-labelled products of enzymic digestions or products of enzymic degradation of standards were applied to Whatman no. 1 chromatography paper and chromatographed with butanol/acetic acid/1 M-NH_3 (2:3:1, by vol.) (Suzuki, 1972). Degraded standards were located by u.v. absorption. ^{35}S-labelled products were located by cutting the strips into 1 cm pieces and counting for radioactivity in a liquid-scintillation spectrometer.

For isolation of undegraded proteoglycans or glycosaminoglycans, samples were spotted on Whatman no. 1 filter paper and chromatographed in a descending system of 1 M-ammonium acetate (pH 7.8)/95% ethanol (2:5, v/v). In this system, proteoglycans and glycosaminoglycans remain at the origin, whereas inorganic [^{35}S]sulphate migrates down the paper. Radioactivity counts at the origin were used to give amounts of ^{35}S-labelled glycosaminoglycan or ^{35}S-labelled proteoglycan in various fractions.

Characterization of the ^{35}S-labelled glycosaminoglycans as chondroitin sulphate, dermatan sulphate or heparan sulphate was performed by sequential use of glycosaminoglycan-degrading enzymes (chondroitinase AC lyase and chondroitinase ABC lyase) as previously described (Kleinman *et al.*, 1975; Yue *et al.*, 1976). Degradation was measured by use of Sephadex G-50 or alternatively by paper chromatography to separate products from undegraded material. Radioactivity was measured in a Searle Delta 300 liquid-scintillation-counting system, with Hydromix (Yorktown Research, Hackensack, NJ, U.S.A.) as the 'scintillation cocktail'.

Results

To determine the specificity of the crude *F. heparinum* enzyme, cells were labelled for 48 h with [^{35}S]sulphate (4.4×10^7 c.p.m./ml of medium), [^{3}H]-leucine (3.3×10^6 c.p.m./ml of medium), [^{3}H]proline (1.64×10^6 c.p.m./ml of medium), [^{3}H]fucose (5.7×10^6 c.p.m./ml of medium) or [^{3}H]glucosamine (8.2×10^6 c.p.m./ml of medium). After exhaustive washing to remove media and labelled substrates, the plated cell monolayers were treated with 1.5 units of *F. heparinum* enzyme for 40 min, followed by trypsin for 10 min. Control plates were treated with phosphate-buffered saline or phosphate-buffered saline and boiled enzyme for 40 min, followed by trypsin for 10 min.

A typical plate yielded approx. 80000 c.p.m. of ^{35}S-labelled glycosaminoglycan in the cell layer fraction (*F. heparinum* enzyme + trypsin treatment),

leaving approx. 4000 c.p.m. in the cell pellet (intracellular) fraction and approx. 4000 c.p.m. still attached to the plate. Results of one experiment (average of three plates) are shown in Table 1. Treatment with the *F. heparinum* enzyme resulted in the release of 77% of the surface-associated ^{35}S-labelled glycosaminoglycan, whereas incubation with the phosphate-buffered saline control resulted in release of only 18% of the ^{35}S-labelled glycosaminoglycan. A phosphate-buffered saline control with boiled *F. heparinum* enzyme was similar to the control with phosphate-buffered saline alone.

F. heparinum enzyme released more than three times as much of the [^{3}H]hexosamine-labelled material as that released from the phosphate-buffered saline control. The released [^{3}H]hexosamine-labelled material co-chromatographed (butanol/acetic acid/1 M-NH_3) with the [^{35}S]sulphate-labelled material released by *F. heparinum* enzyme treatment. Subsequent treatment with trypsin lifted the cells quantitatively and released significant additional amounts of ^3H from the cultures. The trypsin treatment also removed a small additional amount of ^{35}S from the cell surface, representing ^{35}S-labelled glycosaminoglycan not removed by the *F. heparinum* enzyme. Release of ^3H by *F. heparinum* enzyme treatment was not significantly different from release by the phosphate-buffered saline control when the cultures had been labelled with either [^{3}H]leucine, [^{3}H]proline or [^{3}H]fucose.

^{14}C-labelled collagen fibrils were treated with *F. heparinum* enzyme, collagenase, trypsin or phosphate-buffered saline. The *F. heparinum* enzyme (9 units) and phosphate-buffered saline had no effect on either native or denatured [^{14}C]collagen; the collagenase degraded the collagen fibrils; and both collagenase and trypsin degraded heat-denatured fibrils. The *F. heparinum* enzyme (9 units) had no effect on casein.

When crude *F. heparinum* enzyme was incubated with bovine submaxillary mucin in acetate buffer or phosphate-buffered saline buffer, there was no release of sialic acid. Neuraminidase treatment resulted in the release of sialic acid from mucin under the same conditions. When neuraminidase was added to plated cells that had been prelabelled with [^{3}H]glucosamine and [^{35}S]sulphate for 48 h, no net release of either ^3H or ^{35}S was observed.

The appearance on phase microscopy of the cultured cells was not altered by incubation with either *F. heparinum* enzyme or with phosphate-buffered saline. The scheme for testing cell viability of fibroblasts is shown in Fig. 1, performed in triplicate. The plating efficiency and Trypan Blue exclusion confirmed that more than 95% of the fibroblasts were viable after treatment with *F. heparinum* enzyme.

To determine the incubation time required by the *F. heparinum* enzyme for the release of cell-layer material, portions of the buffer were removed from each flask at intervals after enzyme additions to ^{35}S-labelled cell monolayers in triplicate (Fig. 2). Maximum release of 75–78% of the ^{35}S-labelled glycosaminoglycan was achieved by 20–30 min with 1.5 units of *F. heparinum* enzyme. Greater amounts of enzyme did not remove additional ^{35}S-labelled glycosaminoglycan. In subsequent experiments, 40 min was used for the incubation time.

Chondroitin AC lyase (7.5 units) removed 38% of

Table 1. *Release of radioactively labelled substrates by treatment of monolayers with F. heparinum enzyme*
Washed fibroblast monolayers, labelled as indicated, were incubated with *F. heparinum* enzyme followed by trypsin, or with phosphate-buffered saline followed by trypsin as described in the text. Radioactivity released from the cell layer, remaining with the cells, and remaining on the plates after the cells were lifted with trypsin was assayed as described in the text.

	Radioactivity (c.p.m.)				
	Removal by sequential treatment			Remainder	
Cell label	*F. heparinum*	Phosphate-buffered saline	Trypsin	Cells	Plate
[^{35}S]Sulphate	73000	—	16000	4600	3000
	—	18000	61000	4100	5000
[^{3}H]Leucine	27000	—	91000	235000	88000
	—	27000	113000	212000	54000
[^{3}H]Proline	13000	—	24000	34000	13000
	—	11000	35000	27000	19000
[^{3}H]Fucose	1400	—	4300	2700	1400
	—	1800	6900	2000	2000
[^{3}H]Glucosamine	14200	—	15000	34000	2300
	—	4500	25000	33000	3000

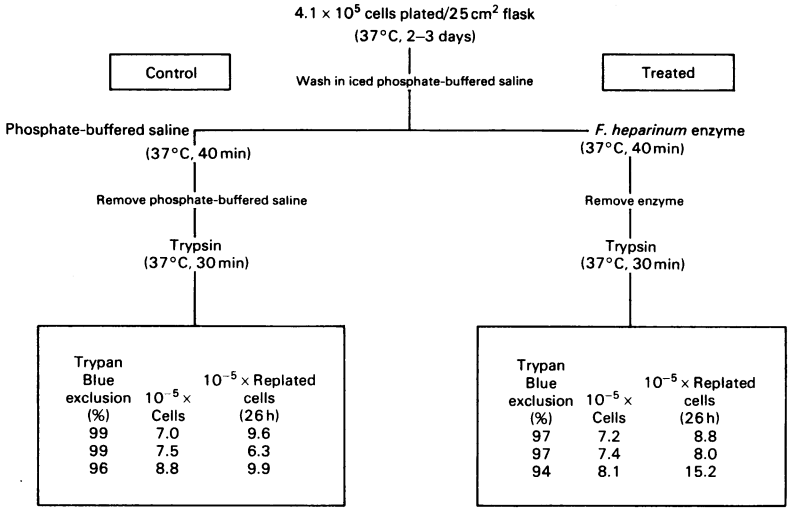

Fig. 1. *Viability of cells after treatment with F. heparinum enzyme*
Cells were grown and treated as shown. Plating efficiency and Trypan Blue exclusion were measured as described in the Methods section.

the cell-layer ^{35}S-labelled glycosaminoglycan. Similar amounts of chondroitin ABC lyase released 50% of the ^{35}S-labelled glycosaminoglycan. The chondroitin ABC lyase had no measurable proteinase activity; the chondroitin AC lyase had slight proteinase activity, but this was not measurable with only 7.5 units. As expected, products of the chondroitin lyase treatments chromatographed as ΔDi-4S and ΔDi-6S with butanol/acetic acid/1 M-NH_3. Treatment with 1.5–3.0 units of *F. heparinum* enzyme produced a mixture of products that moved from the origin of paper chromatograms. With lesser amounts of *F. heparinum* enzyme, ^{35}S-labelled material removed from the cell layers remained at the origin of the chromatograms, indicating lesser degradation. These results are consistent with previous descriptions of the mixture of products formed after treatment of glycosaminoglycans with crude *F. heparinum* enzyme (Korn, 1957; Linker & Hovingh, 1965).

When trypsin was used subsequent to treatment with *F. heparinum* enzyme, the cells were lifted from the plate and an additional 10–20% of the ^{35}S-labelled glycosaminoglycan was removed. This additional ^{35}S-labelled material was characterized by treating the boiled trypsin fraction sequentially with chondroitin lyases and *F. heparinum* enzyme. A mixture of smaller uncharacterized oligosaccharides, chondroitin sulphate and heparan sulphate was found.

Cell monolayers incubated with phosphate-buffered saline alone as a control also showed release of increasing amounts of ^{35}S-labelled glycosaminoglycan with time (Fig. 2). Approx. 10% was released in 30–40 min and 20% in 2 h. One-third of this ^{35}S-labelled glycosaminoglycan was found to be heparan sulphate and two-thirds chondroitin sulphate.

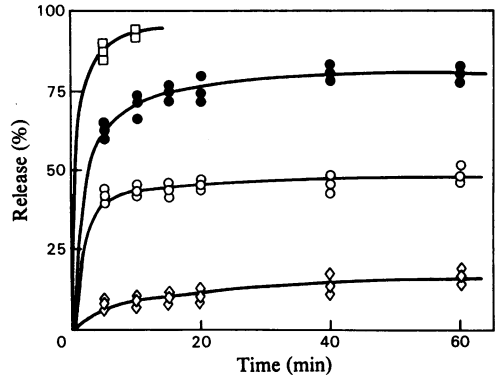

Fig. 2. *Enzymic release of ^{35}S from fibroblast cultures*
Fibroblast monolayers prelabelled with [^{35}S]sulphate were incubated with trypsin (□), *F. heparinum* enzyme (●), chondroitin ABC lyase (○), or phosphate-buffered saline (◇) for various times. Total percentage release of ^{35}S-labelled substances was analysed as described in the Methods section.

Treatment of cells with purified heparinase (provided by Dr. A. Linker) removed approximately half as much ^{35}S-labelled glycosaminoglycan from the plated cell monolayers as that removed by crude *F.*

heparinum enzyme. Most of the ^{35}S-labelled material released by purified heparinase remained at the origin of paper chromatograms, indicating that it was only partially degraded. Gel filtration of the ^{35}S-labelled products of crude *F. heparinum* enzyme treatment and the products of purified heparinase treatment plus similar enzyme treatment of unlabelled carrier heparin is shown in Fig. 3. The crude enzyme degraded all the ^{35}S-labelled glycosaminoglycan to small products, but yielded a mixture of somewhat larger products from the carrier heparin. In contrast, the purified heparinase degraded all the carrier heparin to small products but left half of the ^{35}S-labelled glycosaminoglycan (fractions 14–28) as larger oligosaccharides or polysaccharides. Fractions 14–28 were pooled, dialysed, freeze-dried and then incubated with chondroitin ABC lyase. There

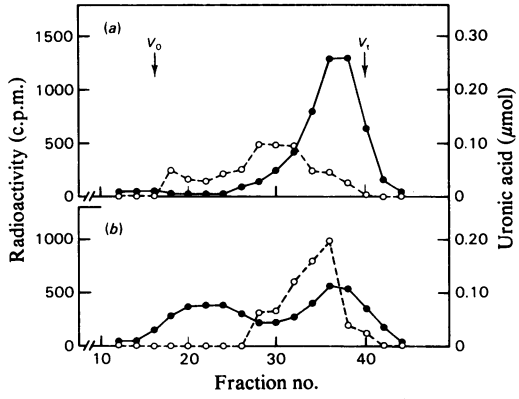

Fig. 3. *Gel chromatography of products after enzyme treatment*

(*a*) ^{35}S-labelled monolayers plus 0.2 mg of heparin were incubated for 40 min with 1.5 units of *F. heparinum* enzyme. (*b*) ^{35}S-labelled monolayers plus 0.2 mg of heparin were incubated for 40 min with 2.6 units (50 μg) of purified heparinase. Products were chromatographed with 0.1 M-LiCl on a column (1 cm × 28 cm) of Sephadex G-75. Fractions (0.6 ml) were collected and analysed for ^{35}S (●) and uronic acid (○).

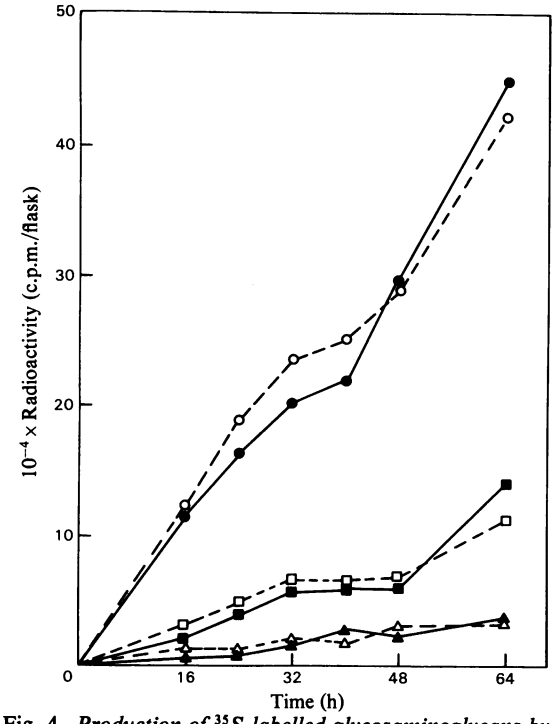

Fig. 4. *Production of ^{35}S-labelled glycosaminoglycans by cell monolayers*

Fibroblast monolayers were treated with 1.5 units of *F. heparinum* enzyme for 40 min. After addition of [^{35}S]sulphate (at zero time), ^{35}S-labelled glycosaminoglycan accumulation was measured in the medium (●), cell-layer fraction (■) and intracellular fraction (▲). Control plates were preincubated with phosphate-buffered saline for 40 min and ^{35}S-labelled glycosaminoglycan accumulation was measured similarly in the medium (○), cell-layer fraction (□) and intracellular fraction (△).

Table 2. *Percentage of dermatan sulphate, chondroitin sulphate and heparan sulphate in various fractions of cell cultures*

The ^{35}S-labelled glycosaminoglycans from Fig. 4 were characterized by sequential use of glycosaminoglycan-degrading enzymes as described in the Methods section. Results represent an average of the ^{35}S-labelled glycosaminoglycans found at the various time points shown in Fig. 4. Total amounts of ^{35}S-labelled glycosaminoglycan in the pellet fractions were too small for accurate characterization.

	Proportion of total glycosaminoglycan (%)		
	Dermatan sulphate	Chondroitin sulphate	Heparan sulphate
Medium			
F. heparinum-treated	35	51	14
Control	28	61	11
Cell layer			
F. heparinum-treated	12	45	43
Control	8	46	46

was no degradation, indicating that these fractions represented heparan sulphate oligosaccharides. Since heparan sulphate contains areas with *N*-sulphated glucosamine, the appearance of some small degradation products (fractions 30–42) was expected.

Production of ^{35}S-labelled glycosaminoglycan by cell cultures subsequent to treatment with *F. heparinum* enzyme is shown in Fig. 4 together with production by control cell cultures that had not been treated with the enzyme. Although there was a suggestion that pretreatment with *F. heparinum* enzyme may have resulted in a small delay in subsequent glycosaminoglycan production, this was not particularly notable. The relative proportions of chondroitin sulphate, dermatan sulphate and heparan sulphate produced in the medium and cell layer are shown in Table 2. The amounts were not significantly altered by pretreatment of the cells with the *F. heparinum* enzyme. Results were similar to previous reports of sulphated glycosaminoglycan production by human skin fibroblast cultures (Kleinman *et al.*, 1975; Truppe & Kresse, 1978; Sjöberg *et al.*, 1979).

Discussion

When cultures of skin fibroblasts are grown in the presence of [^{35}S]sulphate, ^{35}S-labelled proteoglycans are synthesized (Matalon & Dorfman, 1966; Fratantoni *et al.*, 1968; Kleinman *et al.*, 1975; Truppe & Kresse, 1978). Furthermore, the sulphated proteoglycans account for essentially all of the ^{35}S that is incorporated into cell layers and essentially all ^{35}S-labelled macromolecules that subsequently appear in the culture medium (Kleinman *et al.*, 1975). The intracellular pool of free [^{35}S]sulphate is negligible. Thus any removal of ^{35}S from well-washed cell layers corresponds primarily to the removal of the ^{35}S-labelled glycosaminoglycan portions of the ^{35}S-labelled proteoglycans. The incorporation of [^{3}H]glucosamine is less specific, since glucosamine is incorporated as glucosamine or galactosamine into glycoproteins and other substances as well as into the glycosaminoglycan portion of proteoglycans. Incorporation of [^{3}H]fucose is into the terminal sugar of fucose-containing glycoproteins (Buck *et al.*, 1970). Incorporation of labelled amino acids will result in labelling of cellular protein and of extracellular matrix protein, which includes collagen and the protein core of proteoglycans.

Incubation of washed labelled fibroblast monolayers with phosphate-buffered saline for 40min resulted in the release of variable small amounts of labelled material (Table 1). The release of ^{35}S by phosphate-buffered saline was shown to be a time-related (Fig. 3) release of ^{35}S-labelled glycosaminoglycan or proteoglycan, accounting for as much as 20% of the total ^{35}S-labelled glycosaminoglycan of cell layers (Table 1). Release of [^{3}H]serine-labelled, [^{3}H]proline-labelled, [^{3}H]-fucose-labelled or [^{3}H]hexosamine-labelled material generally was approx. 10% of the total radioactivity remaining in the cell layers after these layers had been washed free of medium. This release might reflect material freed by additional washing and/or the release of labelled products into the media through metabolic processes. This was not investigated further.

When washed monolayers were treated with trypsin after the phosphate-buffered saline incubation, cells were quantitatively lifted from the plates. The combined phosphate-buffered saline incubation and the trypsin treatment resulted in solubilization of approx. 90% of the cell-layer ^{35}S (Table 1), which could all be recovered as ^{35}S-labelled glycosaminoglycan. This is in agreement with previous work indicating that almost all the glycosaminoglycans of fibroblast monolayers are in the extracellular matrix or the cell surface (Kleinman *et al.*, 1975; Truppe & Kresse, 1978). Removal of [^{3}H]fucose by phosphate-buffered saline and trypsin was approx. 70% (Table 1), indicating that most of the [^{3}H]fucose-labelled glycoproteins were also in the matrix or on the cell surface. Almost 50% of the [^{3}H]hexosamine-labelled material could be released by trypsin (Table 1), indicating the presence of glycoprotein and proteoglycan in the cell layer. The remaining 50% represented either intracellular material or labelled substances that were insensitive to proteinase. Solubilization of protein by trypsin treatment varied from approx. 35% for [^{3}H]leucine-labelled protein to 50% for [^{3}H]proline-labelled protein (Table 1). The differences in percentage between these two labelled amino acids may reflect differences in composition between proteins that were accessible to the trypsin from those that were not. The release of these various substances by trypsin could be used to determine the amount of extracellular or cell-surface material that could be removed by proteinases.

When washed cell layers were incubated with crude *F. heparinum* enzyme, release of [^{3}H]proline-labelled protein, [^{3}H]leucine-labelled protein or [^{3}H]fucose-labelled glycoprotein was not significantly different from release by incubation with phosphate-buffered saline alone (Table 1). Thus it would appear that the *F. heparinum* enzyme did not contain enzymic material capable of degrading the labelled protein or the labelled glycoprotein. Moreover the *F. heparinum* enzyme was inactive against casein, collagen and denatured collagen. It also contained no measurable sialidase activity.

Incubation of [^{35}S]sulphate-labelled cell layers with the *F. heparinum* enzyme preparation resulted

in the release of almost as much ^{35}S-labelled material as could be released by trypsin. Results with [^{3}H]hexosamine-labelled cells indicated that approximately half as much material could be removed by *F. heparinum* enzyme treatment as by trypsin. This provides a rough estimate of the relative proportions of glycosaminoglycan to glycoprotein in the cell layer, since trypsin will remove glycoprotein as well as glycosaminoglycan. The [^{3}H]hexosamine-labelled products formed by incubation with *F. heparinum* enzyme were similar to the ^{35}S-labelled products obtained similarly. In contrast with the products of trypsin treatment, which have been shown to be intact glycosaminoglycans (Kleinman *et al.*, 1975), the ^{35}S-labelled products and [^{3}H]-hexosamine-labelled products of the *F. heparinum* enzyme digestion were a mixture of small substances (Fig. 3*a*).

The presence of heparan sulphate, dermatan sulphate and chondroitin sulphate in fibroblast cell layers has been described previously (Kleinman *et al.*, 1975; Truppe & Kresse, 1978). Glycosaminoglycan-degrading enzymes, such as chondroitin AC lyase, chondroitin ABC lyase, heparanase (heparitinase) and heparinase, have been used extensively to identify the various types of glycosaminoglycans in cultures (Buonassisi & Root, 1975; Kleinman *et al.*, 1975; Truppe & Kresse, 1978) and elsewhere (Toole & Gross, 1971; Suzuki, 1972; Linker & Hovingh, 1972). We have found that approximately half of the material released by crude *F. heparinum* enzyme could not be released by chondroitin ABC lyase, indicating that it was heparan sulphate. The other half consisted of chondroitin 4-sulphate, chondroitin 6-sulphate and dermatan sulphate. Our results with these enzymes agree with previously published data concerning the glycosaminoglycans of skin fibroblasts.

In contrast with the effects of trypsin treatment, which lifted fibroblast monolayers, the crude *F. heparinum* enzyme had no visible effect on the cells, which remained attached to the plates. Furthermore, incubation with *F. heparinum* enzyme had no noticeable effect on cell viability or capacity for growth (Fig. 1). Production of sulphated glycosaminoglycans subsequent to incubation of fibroblast monolayers with *F. heparinum* enzyme was unaltered. Cells continued to produce sulphated glycosaminoglycans similar in composition and distribution to the sulphated glycosaminoglycans formed by cell monolayers that had not been exposed to the enzyme preparation (Fig. 4).

Thus we have found that a crude enzyme preparation from *F. heparinum* will remove the glycosaminoglycan portion of the proteoglycans of the cell-surface and cell-layer matrix from cultured human skin fibroblast monolayers. We have determined that the general effects of this treatment on growth and on the gross appearance of the cells in culture seem to be negligible. Removal of glycosaminoglycans had no appreciable effect on the subsequent amount or type of glycosaminoglycans produced. This might suggest that in this system proteoglycans of the cell surface and cell-layer matrix do not directly control production of proteoglycans.

It has been suggested that heparan sulphate and other proteoglycans may be involved in cell attachment (Rollins & Culp, 1979). However, our data indicate that removal of most of the heparan sulphate, chondroitin sulphate and dermatan sulphate does not lift the cells from their culture plates, Nevertheless, there was a significant amount (approx. 10%) of cell-layer ^{35}S-labelled proteoglycan still intact after treatment with *F. heparinum* enzyme. It is possible that this material represents proteoglycan protected in an area of cell attachment, which could be sufficient to provide for the attachment.

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